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Biochimie Open 4 (2017) 36–40

http://www.journals.elsevier.com/biochimie-open

Review

The nature of the rate-limiting step of blue multicopper oxidases: Homogeneous studies *versus* heterogeneous

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Received 14 December 2016; accepted 21 January 2017
Available online 3 February 2017

Abstract

Multicopper oxidases (MCOs) catalyzed two half reactions (linked by an intramolecular electron transfer) through a Ping-Pong mechanism: the substrate oxidation followed by the O_2 reduction. MCOs have been characterized in details in solution or immobilized on electrode surfaces. The nature of the rate-limiting steps, which is controversial in the literature, is discussed in this mini review for both cases. Deciphering such rate-limiting steps is of particular importance to efficiently use MCOs in any applications requiring the reduction of O_2 to water.

Keywords: Multicopper oxidase; Rate-limiting step; Homogeneous and heterogeneous studies; Electrochemistry

1. Introduction

Blue multicopper oxidases (MCOs) are ubiquitous and several structures have been resolved for ascorbate oxidase, ceruloplasmin, fungal laccases or bilirubin oxidase [1]. MCOs catalyze two half reactions linked by an intramolecular electron transfer (IET) step:

(i) The substrate oxidation which is specific to each MCOs:
(**Eq. 1**: 4 substrate $\rightarrow$ 4 product + $4e^-$ + $4H^+$) followed by,

(ii) A common reduction of O_2 to water, without producing any toxic oxygen intermediates.
(**Eq. 2**: $O_2 + 4e^- + 4H^+ \rightarrow 2\ H_2O$).

MCOs have been characterized in details [2]. Briefly, the catalytic center is composed of four copper atoms: one T1, one T2 and two T3 which composed the trinuclear center (TNC). They are classified according to their optical and magnetic properties. The T1 has characteristic spectrum in EPR and the blue color of the MCOs under oxidized form is due to the intense absorption band around 600 nm due to the binding T1-S(Cys). The oxidation of substrates occurs on the T1 site whose redox potential varies in function of the axial residue of the pseudo-bipyramid trigonal formed by amino acid residues around the T1. The T2 has also a characteristic feature in EPR. The pair of T3 is silent in EPR, because there are coupled by a strong antiferromagnetic exchange interaction, and has a characteristic absorption band at 330 nm. The T1 accepts the electron from the substrates which are then shuttled to the TNC through ~13 Å of Cys−His residues [3]. The reduction of O_2 into H_2O occurs at the TNC.

MCOs present a great interest for any applications requiring the reduction of O_2 to water, among which enzymatic biofuel cells that may power future implantable medical devices [4,5]. The nature of the rate-limiting step of the global reaction is controversial in the literature either for the enzymatic studies in solution or for the electrochemical studies with immobilized MCOs on electrodes surfaces. For both cases it could be associated to any steps shown in Schemes 1

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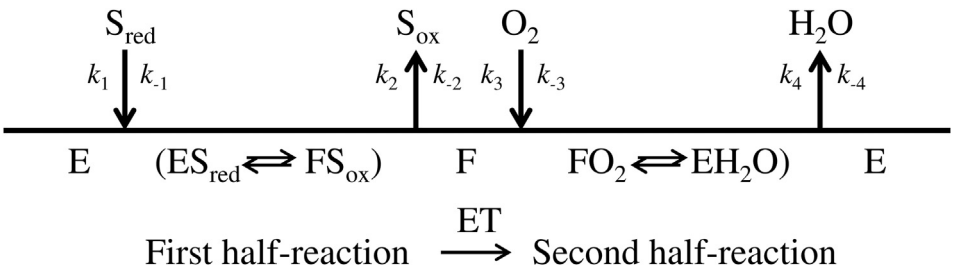
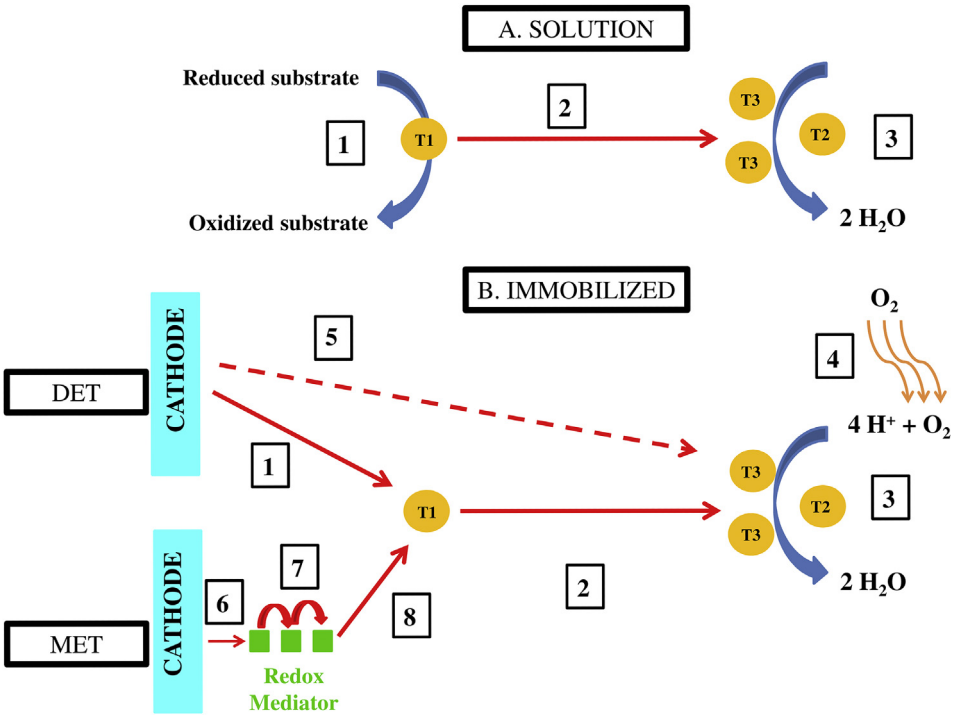
http://dx.doi.org/10.1016/j.biopen.2017.01.001



Scheme 1. General reaction and individual steps of the Ping-Pong Bi Bi system using the Cleland notation. E and F are two different stable enzyme forms. Each substrate addition (S_{red}, O_2) is followed by a product release (S_{ox}, H_2O).



Scheme 2. Different steps for electron transfer for enzymes in solution (A) and immobilized (B) with Direct electron transfer **(DET)** or Mediated electron transfer **(MET)** techniques.

and 2. In addition, in solution it can also be due to any isomerization step or conformational rearrangement [6,7].

In this mini review, we summarized the different arguments through examples of the assignment of the rate-limiting step of MCOs. We will distinguish two types of experiments: in homogenous solution where occur classical redox reactions and immobilized on electrodes surfaces where the enzymes are artificially reduced.

2. Enzymatic studies in solution

To determine the rate-limiting step of the global reaction, the classical method is to compare the k_{cat} determined at steady-state with the individual rate constant of each molecular step. Steady-state kinetic studies showed that MCOs followed BI BI Ping-Pong [6] type mechanism as represented with the Cleland notation (Scheme 1).

Scheme 2A represents the well admitted different steps for electron transfer (ET) for MCOs in solution. Substrate oxidation occurs at the T1 site (**step 1**). The reduction rate constant of the T1 is very efficient and fast as shown by the bleaching of the T1 followed at 600 nm after addition of a reductant [8]. The difference of reduction rate constants observed between different MCOs depends on the rate of intermolecular ET between the substrate and the T1. It also depends on the accessibility of the T1 site of each MCOs [8]. Steady-state kinetic studies on laccases with several substrates have permitted to highlight a linear relationship between the $\log(k_{cat}/K_m)$ and the redox potential difference between the T1 site and the substrate [9]. From those experiments, the rate-limiting step is attributed to the substrate oxidation [2,3,9].

As said by Page and coworkers [10] and according to the Marcus theory [11] the tunneling rates remain higher than catalytic rates (k_{cat}) at typical physiological ΔG (0 to −0.1 eV). The IET (**step 2**) rate constant (*k*IET) from the T1 to TNC has been studied by pulse radiolysis or laser flash photolysis experiments [12–14]. In laccase from *Rhus vernicifera*, *k*IET is >*k*cat so IET is not the rate-limiting step [15].

In human ceruloplasmin, *k*IET is ~*k*cat and IET seems to be associated to the rate-limiting step [12]. In ascorbate oxidase, *k*IET is accelerated in the presence of O_2 [16]. If the IET does not seems to be associated with the rate-limiting step of the overall reaction [16], it appears difficult to generalize for all the MCOs since *k*IET also depends on the presence of O_2 and on the redox state of the TNC [17].

The second half-reaction catalyzed by MCOs (**step 3**) is associated with the O_2 reduction through a two-electron steps. It appears very efficient and not associated to the rate-limiting step. The binding of O_2 leads to the formation of the peroxy intermediate (PI) which decays to the native intermediate (NI) with a fast rate constant (>350 s^{-1}). In NI, the O–O bond is cleaved but two oxygen atoms remain bond to the TNC [3]. In absence of reduced substrate, the NI will turn slowly into the oxidized resting form of the enzyme.

3. Electrochemical studies

Electrochemistry and in particular protein film electrochemistry [18] is a powerful tool to decipher ET within immobilized MCOs and to elucidate the different redox state of MCOs [19,20]. Two techniques are widely used to electrically contact MCOs on electrode surfaces: Direct Electron Transfer (DET, Scheme 2B) where the enzyme is directly connected to the electrode surface and Mediated Electron Transfer (MET, Scheme 2B) where a redox mediator is used to shuttle electrons from the electrode to the enzymes. We will only consider the case of 4 copper containing MCOs displaying an activity towards the reduction of O_2. While in homogeneous solution the rate-limiting step has often been attributed to the rate of oxidation of substrates, in electrochemistry, the rate-limiting step depends on numerous parameters.

Once the MCOs are immobilized onto electrodes surfaces (Scheme 2B), the limiting step can be **step 1** if enzymes are not properly oriented on the electrode surface or if the applied electrode potential is not reducing enough [3]. To ensure fast ET, the distance between the T1 and the electrode surface needs to be as short as possible. For example, when laccases were properly oriented on steroid modified carbon nanotubes, it was reported that the heterogeneous ET rate between the T1 and the electrode could be as high as 3000 s^{-1} [21]. In addition, immobilized enzymes have to be as stable as possible on the electrode surfaces. Leaching or reorganization of the enzyme on the electrode surface may also have a significant impact on step 1 [22]. The IET (**step 2**) between the T1 site and the TNC can also be the rate-limiting step. For example, dos Santos et al. [23] studied the behavior of BOD from *Myrothecium verrucaria* covalently immobilized on graphite electrode through diazonium coupling. They demonstrated that at pH 8, the limiting step was the IET while at pH 5 it was step 1. **Step 3**, which represents the reduction of O_2 by the T2/T3 is usually a fast process with apparent bimolecular constants ~10^6 $M^{-1}\cdot s^{-1}$ and is generally not considered as a limiting step [24]. Finally, the diffusion of O_2 to the electrode surface (**step 4**) can also be the rate-limiting step particularly in electrochemistry. Very recently, it has also been hypothesized that by an adequate immobilization of bilirubin oxidase it would be possible to short circuit the IET and inject directly electrons to the TNC (**step 5**) [25].

In addition to the above mentioned rate-limiting steps, in MET, three additional can be identified: **steps 6** and **8** which corresponds respectively to the ET from the electrode to the redox mediator and from the redox mediator to MCOs. It will also of course depend if the redox mediator is soluble or immobilized onto the electrode surface. In the particular case where redox polymers are used, such as osmium based redox polymers for example, the ET within the redox mediators may also be a limiting step (**step 7**) [26,27].

4. Discussion

From the literature, two consensuses emerge from the study of the rate limiting steps in MCOs. In homogeneous conditions, the rate-limiting step is generally attributed to the substrate oxidation. However, there is at least one example where the IET seems to be associated with the rate-limiting step in ceruloplasmin. Of course, it is not excluded that differences observed in homogenous solution are not due to different experimental conditions. pH, salts, temperature, etc… may influence the enzymes kinetics and therefore the nature of the rate limiting step [28]. Either in homogeneous or heterogeneous conditions the reduction of O_2 is not associated with the rate-limiting process.

In heterogeneous conditions, most of the time the rate limiting step is different from the one observed in homogenous solution. Even though it is most likely due to the ET to the T1, it is much more complicated to definitely conclude about it. For immobilized enzymes, several interrelated parameters must be taken into account to identify the nature of the rate-limiting step of the reaction: the intrinsic nature of the enzyme, its method of immobilization on the electrodes surfaces, its orientation, the diffusion of substrate or product, the electrode materials and geometry, the presence or absence of a redox mediator, the efficiency of the redox mediators and etc…. In addition, conformational dynamics of enzyme and ET are often linked [29] and the properties of confined or unconfined enzymes within an electrode may be different. So it cannot be excluded that the differences observed between enzymes in solution or immobilized are due to differences in protein motion, particularly when porous electrodes are used.

Different analytical tools have been developed to elucidate the nature of rate limiting steps of immobilized enzymes. Protein thin film voltammetry has been used with success to decipher mechanism of immobilized enzymes in DET [18]. Due to the complexity of numerous enzymatic systems, and of the numerous interrelated parameters to be taken into account, the combination of different analytical tools is also explored. For example, Lojou et al. combined surface plasmon resonance, polarization-modulated infrared reflection absorption spectroscopy to correlate the loading, conformation and activity of Bilirubin oxidase immobilized on carbon nanotubes electrode [30]. Blanford et al. used Quartz Crystal

Microbalance with Dissipation and electrochemistry to correlate catalytic activity and enzymes motion on electrodes surfaces [31]. Atanassov et al. used molecular docking simulations and electrochemistry to gain insight into interaction between quinones and immobilized glucose dehydrogenase on electrodes surfaces [32]. In the same time, a more engineering approach is used to improve ET rate using directed evolution, semi-rational or rational methods [33]. Recently, interesting strategies have been developed and consist in screening enzymes directly on electrode surfaces to maximize orientation and ET [34,35]. An example of a successful combined approach is the cofactor redesign of glucose oxidase which minimizes the competition between O_2 and redox mediator for the electrons generated by the oxidation of glucose [36].

5. Conclusion

In this mini review, we discussed the rate limiting steps of MCOs both in solution and immobilized on electrodes surfaces. Even though some trends may appears, predicting the behavior of immobilized MCOs on electrode surfaces from the results obtained in solution remains elusive. Deciphering the kinetic mechanism of immobilized MCOs is of tremendous importance to develop, for example, new generation of enzymatic biofuel cells which are currently limited by the MCOs cathodic enzyme. More generally, enzymes are not naturally designed to operate immobilized on electrode surface. If the ability to tailor enzymes at will for biotechnological applications in homogeneous solution has been routinely used with success [37–39], redesigning functional enzymes for bioelectrochemical applications is a challenging task. Due to the numerous interrelated parameters, elucidating the rate limiting steps of immobilized enzymes with the objective to improve them will require new approaches by combining the latest experimental techniques and theoretical methods [40].

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank la Région Aquitaine, University of Bordeaux and ANR (RATIOCELLS -ANR-12-BS08-0011-01) for financial supports. Elodie Roussarie thanks the Ministry of National Education, Research and Technology for a MENRT research grant.

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